

Full Length Research Paper

# Genetic diversity analysis of Iranian citrus varieties using micro satellite (SSR) based markers

M. Jannati<sup>1</sup>, R. Fotouhi<sup>1</sup>, A. Pourjan Abad<sup>2\*</sup> and Zivar Salehi<sup>3</sup>

<sup>1</sup>Department of Horticulture, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran.

<sup>2</sup>Agric-Natural Research Center of Yazd, Genomics Laboratory, Yazd, Iran.

<sup>3</sup>Department of Biology, Faculty of Sciences, University of Guilan, Rasht, Iran.

Accepted 8 June, 2009

Fifteen SSR Primer Pairs were used to estimate the level of polymorphism among 23 Citrus genotypes and four natural hybrids or bud mutation was selected from Kotra Germplasm Bank (IRAN). All fifteen loci assayed in citrus plant possessed a high level of polymorphism, with the number of alleles per locus ranging from 4 in TAA41 to 12 at CAT01, ATC09, AG14 ( an average, 8.27 alleles were detected per locus ). Cluster analysis with SSR markers resulted in 2 cluster groups: Group A: Yuzo and *Poncirus*. Group B: There are three separate subgroups within Group B; (i) genus *Fortunella sp* (ii) Mandarin subgroup: *Citrus reticulata* (*Citrus clemantin*), *Citrus sinensis* (Pineapple, Washington Navel), Natural types (Siahvaraz, Shalmahaleh, Moallemkoh and Kotra 4 hybrids) and (iii) *Citrus Limon* (Amol lemon - pear, Eureka, Rough Lemon), *Citrus aurantifolia*, *Citrus aurantium*, *Citrus medica* and *Citrus grandis*. Microsatellite analysis clustered citron and sour orange cv cluster but these taxa were quiet distant from *Fortunella SP*.

**Key words:** *Citrus*, microsatellite, phylogeny, polymorphism, germplasm bank, genetic diversity.

## INTRODUCTION

*Citrus* plants are cultivated in the North and South of IRAN. Little is known about the genetic variability of Iranian cultivated citrus germplasm collection. Microsatellite or SSR (Simple Sequence Repeat) markers are co-dominant, multiallelic, highly polymorphic genetic markers and appropriate for genetic diversity studies.

*Citrus* Cultivated since ancient times in its centre of origin in south eastern Asia, citrus production has spread over the centuries into most areas that have a suitable climate (Webber et al., 1967). Today citrus is one of the most widely cultivated fruit in the world, and most major production areas are far removed from the original areas. Different *Citrus* species widely grown in more than 50 countries in the world. World production is increasing and has reached 70 million tones, according to FAO (Orford et al., 1995).

Citrus taxonomy and phylogeny, however, are very complicated, controversial and confusing, mainly due to

sexual compatibility between *Citrus* and related genera, the high frequency of bud mutations and the long history of cultivation and wide dispersion. Citrus varieties show diversity in their morphological traits such as size and shape of canopy, color, size, type and ripening season of the fruits and the number of seeds per fruit (Orford et al., 1995).

In the past, studies on relationships between genera and species were carried out based mainly on morphological and characteristics. Numerous classification systems have been formulated, among which those of Swingle (1943) and Tanaka (1977) have been the most widely accepted. Even these two researchers, however, have quite different concepts with respect to species classification, as Swingle included only 16 species in Citrus while Tanaka described 162. Later phylogenetic analysis by Scora (1975) and Barrett and Rhodes (1976) suggested that there were only 3 true species within the cultivated *Citrus*, that is, citron (*Citrus medica* L.), mandarin (*C. reticulata* Blanco) and pummelo [*C. grandis* (L.) Osb.] (in 1988 Scora added another true species: *C. halimii* Stone). In addition, other genotypes were derived from hybridization between these true species (Scora, 1988). More recently, biochemical data (Potvin et al.,

\*Corresponding author. E-mail: [ali1360405@gmail.com](mailto:ali1360405@gmail.com). Tel: +98 351 8249901. Fax: +98 3518247439

**Table 1.** Cultivars, species, natural hybrids and bud mutation used in this study.

Common name	Type of cultivar	
	Genus and species	Natural hybrid or bud mutation
Yuzo	<i>Citrus junos</i> Sieb	
Trifoliolate Orange	<i>Poncirus trifoliata</i>	
Kumquat	<i>Fortunella Sp.</i>	
Clemantin	<i>Citrus reticulata</i> Blanco	
Satsuma Mandarin	<i>Citrus unshiu</i> Marcovich	
King or Sweet Orange	<i>Citrus nobilis</i>	
Pineapple Orange	<i>Citrus sinensis</i> (L.) Osbeck	
Washington Navel Orange	<i>Citrus sinensis</i>	
Siahvaraz	<i>Citrus sinensis</i>	Bud mutation
Moallemkoh	<i>Citrus sinensis</i>	Bud mutation
Kotra 2 - 4	-	Natural hybrid
Kotra 1 - 4	-	Natural hybrid
Shalmahaleh	-	Natural hybrid
Mexican Lime	<i>Citrus aurantifolia</i>	
Sweet Lime	<i>Citrus aurantifolia</i> (L.)	
Sour Orange	<i>Citrus aurantium</i> (L.)	
Amol Lemon-Pear	Near to <i>Citrus limon</i>	Bud mutation or natural hybrid
Citrus King (Pumelo)	<i>Citrus grandis</i> (L.) Osb	
Cluster Lemon	<i>Citrus limon</i> Burn.f	
Rough Lemon	<i>Citrus limon</i> Burn.f	
Eureka Lemon	<i>Citrus limon</i>	
Etrag Citron	<i>Citrus medica</i> (L.)	
Nova	Near to <i>Citrus reticulata</i>	Complex hybrid of Mandarin

1983), protein electrophoresis (Handa et al., 1986), isozymes (Torres et al., 1978; Fang et al., 1993; Herrero et al., 1996), microsatellites (Kijas et al., 1995), organeller genome analysis (Green et al., 1986; Yamamoto et al., 1993) and (Fang et al., 1997; Fang et al., 1998) have been used to examine relationships among *Citrus* taxa. Microsatellites, or simple sequence repeats (SSRs), are short sequence elements composed of tandem repeat units one to seven base pairs (bp) in length (Tautz, 1989). These repeats sequences have been shown to be highly polymorphic within and between species, a property that has permitted their application as molecular markers in population genetics (Goldstein et al., 1999), systematics (Goldstein and Pollock, 1997), and genome mapping (Weissenbach et al., 1992).

Microsatellites are present in high numbers in mammals and in plant genomes too, but appear to be less abundant than in mammalian or insect systems (Van Treuren et al., 1997). Thomas et al., 1994 distinguished 20 grapevines varieties by using four microsatellite loci. They proposed the use of microsatellites for establishing an international database for description of grapevine varieties, based on its high level of polymorphism, co-dominance, simplicity of analysis and repeatability (Thomas et al., 1994).

Little is known about the genetic variability of the Iranian Citrus Germplasm Collections. We investigated the phylogenetic relationships among 23 citrus plants of the Kotra Germplasm Bank (IRAN) and studied the origin of some important Citrus species of this Collection, using microsatellites markers.

## MATERIALS AND METHODS

### Plant materials and DNA isolation

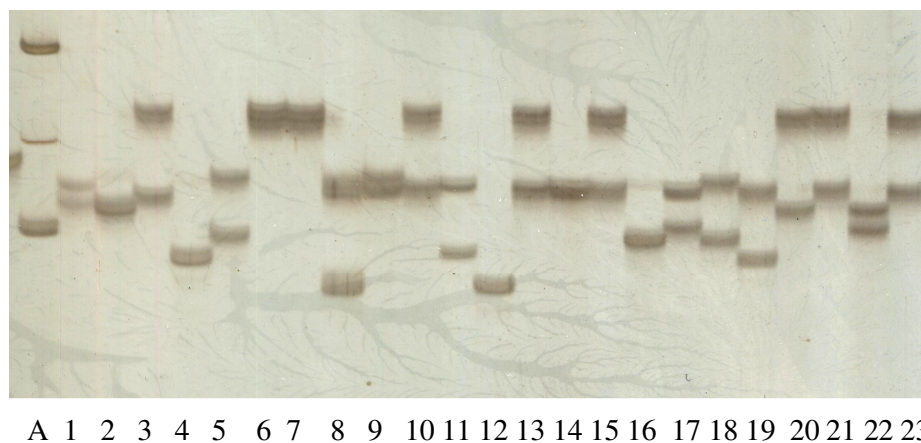
In this study, 23 cultivars, species, natural hybrids or bud mutations were used (Table 1), which held at Germplasm Collection of Kotra (Iran). From each accession, 50 mg of young expanding leaves were collected and stored at -80°C before DNA isolation. Genomic DNA was isolated from leaf samples in accordance with the CTAB (Hexadecyltrimethyl ammonium bromide) method described by Doyle and Doyle (1987). DNA was quantified by comparing it with lambda DNA (Promega Corporation, Madison, Wis) on ethidium bromide stained agarose gels.

### PCRs and electrophoresis

Fifteen primer pairs (TAA15, TAA27, TAA41 CAC23, CAC15, CAC33, CAC39, CCT01, CAT01, ATC09, AG14, CTT01, CT21, TC26 and CT19) (MWG Biothec, Germany) were used in this

**Table 2.** SSR loci characterization, size of amplified fragments; primer pairs repeat motifs and total number of alleles.

Locus code	Repeat	Forward Primer	Reverse Primer	Alleles	Size range (bp)
TAA15	TAA	GAAAGGGTACTTGACCAGGC	CTTCCCAGCTGCACAAGC	5	123 - 130
TAA27	TAA	GGATGAAAAATGCTCAAATG	TAGTACCCACAGGGAAGAGAGC	10	158 - 230
TAA41	TAA	AATGCTGAAGATAATCCGCG	TGCCTTGCTCTCCACTCC	4	242 - 265
CAC23	CAC	ATCACAATTACTAGCAGCGCC	TTGCCATTGTAGCATGTTGG	6	105 - 135
CAC15	CAC	TAAATCTCCACTCTGCAAAAGC	GATAGGAAGCGTCGTAGACCC	10	135 - 190
CAC33	CAC	GGTGATGCTGCTACTGATGC	CAATTGTGAATTTGTGATTCCG	8	77 - 109
CAC39	CAC	AGAAGCCATCTCTTCTGCTGC	AATTCAGTCCCATTCCATTCC	6	120 - 165
CCT01	CCT	TCAACACCTCGAACAGAAGG	CCCACATGCTAGCACAAAGA	8	93 - 119
CAT01	CAT	GCTTTGATCCCTCCACATA	GATCCCTACAATCCTTGGTCC	12	138 - 172
ATC09	ATC	TTCCTTATGTAATTGCTCTTTG	TGTGAGTGTGTTGTGCGTGTG	12	130 - 210
AG14	AG	AAAGGGAAAGCCCTAATCTCA	CTTCCTCTTGCGGAGTGTTT	12	110 - 172
CTT01	CTT	TCAGACATTGAGTTGCTCG	TAACCACTTAGGCTTCGGCA	7	226 - 252
CT21	CT	CGAACTCATTAAAAGCCGAAAC	CAACAACCACCACTCTCACG	8	130 - 170
TC26	TC	CTTCTCTTGCGGAGTGTTT	GAGGGAAAGCCCTAATCTCA	7	93 - 119
CT19	CT	CGCCAAGCTTACCACTCACTAC	GCCACGATTTGTAGGGGATAG	9	175 - 205



**Figure 1.** Microsatellite polymorphism (locus CAC15) A is size marker. Lane 1 = Yuzu; Lane 2 = Rough Lemon; Lane 3 = Clemantin; 4 = Etrag Citron; 5 = Sour Orange; 6 = Satsuma Mandarin; 7 = Kumquat; 8 = Amol Lemon-Pear; 9 = Siahvaraz; 10 = Cluster lemon; 11 = Trifoliolate Orange; 12 = Pinapple Orange; 13 = Citrus king (Pumelo); 14 = Washington Navel Orange; 15 = Eureka Lemon; 16 = Sweet Lime; 17 = cluster sour orange; 18 = Moallemkoh; 19 = Shalmahaleh; 20 = Kotra 2-4; 21 = Nova; 22 = Kotra 1-4; 23 = Mexican Lime.

research (Table 2). PCRs were performed in a final volume of 10  $\mu$ L, containing the following: 20 mmol Tris-HCl/L (pH 8.4); 50 mmol KCl/L; 1.5, 2.5, or 5 mmol MgCl<sub>2</sub>/L, depending on the primers; 0.1 mmol/L of each dNTP (deoxynucleoside triphosphate); 0.8  $\mu$ mol/L of each primer; 20 ng of genomic DNA; and 1 U *Taq* polymerase (Invitrogen, Carlsbad, Calif). The following temperature profile was used: 95°C for 1 min, then 35 cycles of 94°C for 45 s, 45 – 63°C for 45 s and 72°C for 75 s), ending with 72°C for 7 min (Progene; Techne, Cambridge, UK). PCR products were separated by electrophoresis in 6% acrylamide gels, stained with ethidium bromide (0.8  $\mu$ g/mL), using 1 $\times$  TBE (89 mmol Tris/L, 89 mmol boric acid/L and 2 mmol EDTA/L (pH 8.0) buffer and visualized under ultra-violet light. Molecular sizes of the amplified fragments were estimated using a 100-bp ladder (Invitrogen) (Soriano et al., 2005) (Figure 1).

#### PIC (polymorphism information content) value

In order to determine the informativeness of the microsatellites, the PIC values were calculated. PIC value was calculated according to the formula:

$$PIC = \sum 1 - P_{ij}^2$$

#### Polymorphism analysis

For a single locus, the presence of amplified fragments was scored as 0.5 if the Individual was heterozygous, 1 if it was homozygous, and 0 if the allele was not present. According to these observations,

**Table 3.** PIC value of different Iranian *Citrus* germplasm.

SSR	PIC*					Mean PIC
	Natural hybrids	Grape fruit	Lemons	Mandarins	Citrus	
TAA15	0.69	0.50	0.50	0.50	0.50	0.65
TAA27	0.62	0.75	0.76	0.72	0.72	0.85
TAA41	0.0	0.81	0.48	0.55	0.50	0.52
CAC23	0.0	0.38	0.58	0.56	0.0	0.71
CAC15	0.72	0.63	0.85	0.83	0.72	0.86
CAC33	0.65	0.61	0.68	0.54	0.52	0.78
CAC39	0.67	0.50	0.50	0.69	0.50	0.72
CCT01	0.69	0.62	0.61	0.52	0.41	0.75
CAT01	0.85	0.81	0.80	0.87	0.27	0.89
ATC09	0.67	0.63	0.69	0.53	0.50	0.76
AG14	0.59	0.38	0.85	0.67	0.0	0.87
CTT01	0.58	0.53	0.56	0.68	0.53	0.74
CT21	0.81	0.75	0.78	0.67	0.50	0.73
TC26	0.89	0.50	0.82	0.67	0.0	0.83
CT19	0.71	0.76	0.78	0.59	0.48	0.79
<b>Mean PIC</b>	0.64	0.61	0.68	0.63	0.41	

\*Polymorphic information content.

a similarity matrix was generated using the Nei's genetic distance (Nei, 1972). Similarity data were processed through the unweighted pair-group method (UPGMA) cluster analysis conducted using NTSYS program (Exeter Software, Setauket, N.Y.) (Rohlf, 1993), program, applying the Jaccard (1908) and Dice (Sneath and Sokal, 1973) coefficients. The goodness of fit measured by the cophenetic correlation unweighted pair-group method, arithmetic average (UPGMA) cluster analysis and finally depicted in a dendrogram (Figure 1).

## RESULTS

### Microsatellite polymorphism and accession variability

Microsatellites analysis clustered Citron and sour orange cv cluster but these taxa were quiet distant from *Fortunella SP.*

The present study showed the utility of microsatellite markers for the detection of polymorphisms among the Iranian citrus germplasm. The identification of similarity group could be useful for the selection of parental plants to be used in the breeding programs.

All fifteen loci assayed in citrus plant possessed a high level of polymorphism, with the number of alleles per locus ranging from 4 in TAA41 to 12 at CAT01, ATC09, AG14 (an average, 8.27 alleles were detected per locus).

Where  $P_{ij}$  is the frequency of the  $j$ th microsatellite allele for loci. This value is referred to as heterozygosity and gene diversity (Weir 1990, Anderson et al, 1993).

The most highly polymorphic loci were: CAT01 (12 alleles, PIC=0.89), AG14 (12 alleles, PIC = 0.87), TAA27

(10 alleles, PIC = 0.85) (Table 3).

### Cluster analysis

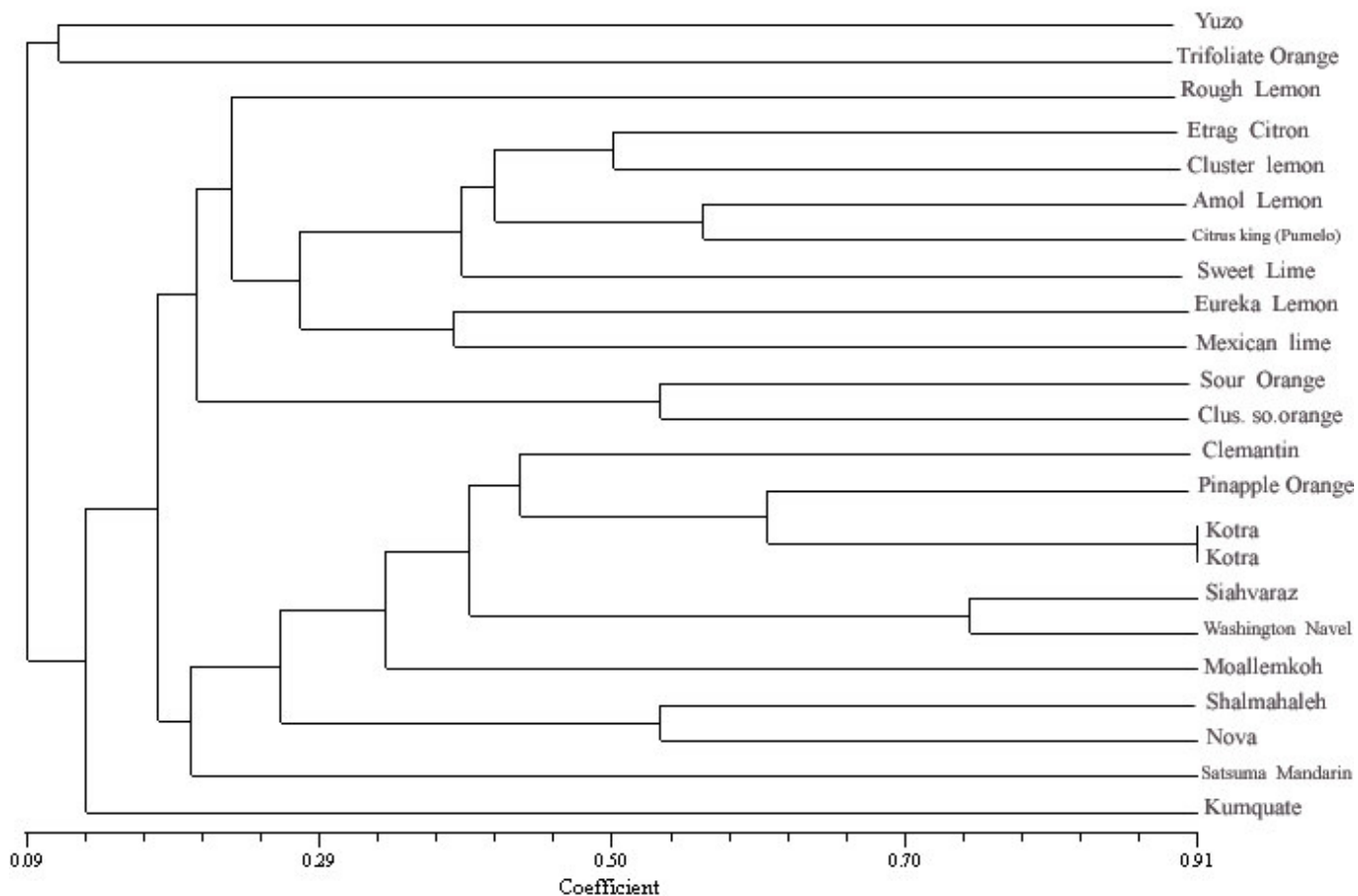
UPGMA cluster analysis of the similarity matrix obtained from 23 SSR alleles (Nei 1972) resulted in a dendrogram of genetic relationships that grouped cultivars in agreement with their geographic origins and pedigrees (Figure 2), producing 2 main clusters. The first cluster Included Yuza and *Poncirus*. The second cluster was subdivided into 3 sub-clusters (i) genus *Fortunella sp* (ii) Mandarin subgroup: *Citrus reticulata* (*Citrus clemantin*), *Citrus sinensis* (Pineapple, Washington Navel), Natural types (Siahvaraz, Shalmahaleh, Moallemkoh and Kotra 4 hybrids) and (iii) *Citrus limon* (Amol lemon-pear, Eureka, Rough Lemon), *Citrus aurantifolia*, *Citrus aurantium*, *Citrus medica* and *Citrus grandis*.

Microsatellite analysis clustered Citron and sour orange cv cluster but these taxa were quiet distant from *Fortunella SP.*

## DISCUSSION

The transportability of the microsatellites among species belonging to different genera or even families has been previously reported (Dirlewanger et al., 2002).

In our work microsatellite markers were used to study genetic diversity in 23 citrus plants of the Kotra Germplasm Collection, IRAN (Yuza, Rough Lemon, Clemantin, Etrag Citron, Sour Orange, Satsuma Mandarin, Kumquat,



**Figure 2.** Dendrogram of the 23 citrus cultivars included in this study generated by unweighted pair-group method (UPGMA) cluster analysis from the similarity matrix obtained using Nei's (1972) genetic distance.

Amol Lemon-Pear, Siahvaraz; Cluster lemon, Trifoliolate Orange, Pineapple Orange, Citrus king (Pumelo), Washington Navel Orange, Eureka Lemon, Sweet Lime, Moallemkoh, Shalmahaleh, Kotra 2 - 4, Nova, Kotra 1 - 4, Kumquate, Mexican Lime and Sour Orange var. Cluster). According to Wang et al. (1994), in plant nuclear DNA the dinucleotides sequence (AT) $n$  is the most abundant, followed by (A) $n$ /(T) $n$  and (AG) $n$ /(CT) $n$ . In our experiments, CAT01, ATC09, AG14 gave excellent fingerprint patterns, suggesting that these repeats are abundant in citrus plants. In the study of Gulsen and Roose (2001) cpDNA indicated that *Fortunella sp* had totally different microsatellite patterns from the other taxa analysed. Although *Fortunella* is well differentiated from *Citrus* on the basis of detailed morphological studies, apparently there has not been the same level of divergence at the molecular level. Our experiments indicated that the genus *Citrus* is quite distant from the related genus *Poncirus*. Kotra 1 - 4 and Kotra 2 - 4 probably originated as nucellar seedling from the same tree. Siahvaraz has a much similarity to Washington Navel orange and probably originated from bud mutation. Shalmahaleh is a natural hybrid and has a greater similarity to Nova. Moallemkoh has

a similarity to Washington navel Orange and is probably hybrid between them or as a bud mutation. Shalmahaleh, Nova, Etrag Citron and Citrus King (Pumelo) are very similar and Shalmahaleh is apparently as a hybrid origin, most probably of Nova and Etrag Citron or Nova and Citrus King (Pumelo). Amol Lemon-Pear is probably derived from hybridization between Rough Lemon and Citrus King and has a similarity to them.

The percentage of PIC (polymorphic Information Content) in lemon, Mandarin, Grapefruit, Natural hybrid and sweet orange were 0.68, 0.63, 0.61, 0.64, 0.41 as observed by Novelli et al. (2000).

Heterozygosity is important to both natural and cultured populations because (1) it provides a large spectrum of genotypes for adaptive response to changing conditions and (2) heterozygous individuals usually are superior to less heterozygous individuals in many economically important characteristics like growth, fertility and disease resistance. A set of informative SSR markers detected considerable levels of genetic variability in the Iranian citrus germplasm. The identification of similarity group could be useful for the selection of parental plants to be used in the breeding programs.

## ACKNOWLEDGEMENTS

We thank Prof. M. L. Roose for primers, Mr. Amin Ramezani for his helpful comments that improved the manuscript and Mr. Dalir Seffat for his technical assistance.

## REFERENCES

- Anderson JA, Churchill GA, Autrique JE, Tanksley SD, Sorreles ME (1993). Optimizing paternal selection for genetic linkage maps. *Genome* pp: 36:181-186.
- Barrett HC, Rhodes AM (1976). A numerical taxonomic study of affinity relationship in cultivated *Citrus* and its close relatives. *Systematic Bot.*, 1:105-136.
- Dirlwanger E, Cosson P, Tavaud M, Aranzana MJ, Poizat C, Zanetto A, Arus P, Laigret F (2002). Development of microsatellite markers in peach [*Prunus persica* (L.) Batsch] and their use in genetic diversity analysis in peach and sweet cherry (*Prunus avium* L.). *Theor. Appl. Genet.* 105: 127–138.
- Doyle JJ, Doyle JL (1987). A rapid isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19: 11–15.
- Fang DQ, Roose ML, Krueger RR, Federici CT (1997). Fingerprinting trifoliate orange germplasm accessions with isozymes, RFLPs, and inter-simple sequence repeat markers. *Theor. Appl. Genet.* 95: 211 - 219.
- Fang DQ, Zhang WC, Xiao SY (1993). Studies on taxonomy and evolution of *Citrus* and its related genera by isozyme analysis. (in Chinese with English abstract). *Acta Phytotaxon Sinica*, 31: 329-352.
- Fang DQ., Krueger RR, Roose ML (1998). Phylogenetic relationships among selected *Citrus* germplasm accessions revealed by inter-simple sequence repeat (ISSR) markers. *J. Am. Soc. Hort. Sci.* 123: 612-617.
- Goldstein DB, Pollock DD (1997). Launching microsatellites: A review of mutation processes and methods of phylogenetic inference. *J. Hered.* 88: 335-342.
- Goldstein DB, Roemer GW, Smith DA, Reich DE, Wayne RK (1999). The use of microsatellite variation to infer population structure and demographic history in a natural model system. *Genetics*, 151: 797-801.
- Green RM, Vardi A, Galun E (1986). The plastome of *Citrus*. Physical map, variation among *Citrus* cultivars and species, and comparison with related genera. *Theor. Appl. Genet.*, 72: 170-177.
- Handa T, Ishizawa Y, Oogaki C. 1986. Phylogenetic study of Fraction I protein in the genus *Citrus* and its close related genera. *Jpn J Genet* 61:15-24.
- Herrero, R., M.J. Asins, E.A. Carbonell and L. Navarro. 1996. Genetic diversity in the orange subfamily Aurantioideae. I. Intraspecific and intragenus genetic variability. *Theor. Appl. Genet.* 92: 599-609.
- Soriano JM, Romero C, Vilanova S, Llácer G, Badenes ML (2005). *Genome* 48: 108–114.
- Kijas JMH, Fowler JCS, Thomas MR (1995). An evaluation of sequence tagged microsatellite site markers for genetic analysis within *Citrus* and related species. *Genome* 38: 349-355.
- Nei M (1972). Genetic distance between populations. *Am. Naturalist*, 106: 283 – 292.
- Orford SJ, Steele Scott N, Timmis JN (1995). A hypervariable middle repetitive DNA sequence from citrus. *Theor. Appl. Genet.*, 91:1248-52.
- Potvin C, Bergeron Y, Simon JP (1983). A numerical taxonomic study of selected *Citrus* species (Rutaceae) based on biochemical characters. *Syst. Bot.* 8:127-133
- Scora RW (1975). On the history and origin of *Citrus*. *Bul. Torrey Bot. Club*, 102: 369-375
- Scora RW (1988). Biochemistry, taxonomy and evolution of modern cultivated *Citrus*. In: Goren R, Mendel K (eds). *Proc. 6<sup>th</sup> Int'l. Citrus Congr. Margraf Pub1., Weikersheim, Germany.* 1: 277-289.
- Scott NS (eds) *Techniques on gene diagnosis and breeding in fruit trees.* Fruit Trees Research Station, Okitsu, Japan, pp: 39-46
- Swingle WT (1943). The botany of *Citrus* and its wild relatives of the orange subfamily, In: Webber HJ, Batchelor LD (eds.). *The Citrus industry, History, botany, and breeding.* Univ. California Press, Berkeley, Calif., 1: 129-474
- Tanaka T (1977). Fundamental discussion of *Citrus* classification. *Studia Citrologia*, 14: 1-6
- Tautz D (1989). Hypervariability of simple sequence repeats as a general source for polymorphic DNA markers. *Nucleic Acids Res.*, 17: 6463 - 6471
- Thomas MR, Scott NS (1994). Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequence-tagged sites (STSs). *Theor. Appl. Genet.* 86, 985-990
- Thomas MR, Cain PNS (1994). DNA typing of grapevines: a universal methodology and database for describing cultivars and evaluating genetic relatedness. *Plant Mol. Biol.* 25: 939-949
- Torres AM, Soost RK, Diedenhofen U (1978). Leaf isozymes as genetic markers in *Citrus*. *Am. J. Bot.* 65: 869 - 881
- Van Treuren R, Kuittinen H, Karkkainen K, Baena-Gonzalez E, Savolainen O (1997). Evolution of microsatellites in *Arabidopsis petraea* and *Arabidopsis lyrata*, outcrossing relatives of *Arabidopsis thaliana*. *Mol. Biol. Evol.* 14: 220-229
- Webber HJ, Reuther W, Lawton HW (1967). History and development of the citrus industry, In: Reuther W., Webber HJ, Batchelor LD (Eds). *The Citrus industry.* University of California, Berkeley, 1: 1-39
- Weir BS (1990). Genetic data analysis. Methods for discrete genetic data. Sinauer associates, Sunderland, MA.
- Weissenbach X, Gyapay G, Dib C, Vignal A, Morisset J, Millasse P (1992). A second-generation linkage map of the human genome. *Nature* 359: 794-801
- Yamamoto M, Kobayashi S, Nakamura Y, Yamada Y (1993). Phylogenetic relationships of *Citrus* revealed by diversity of cytoplasmic genomes. In: Hayashi T, Omura M